Quorum sensing in Streptomyces coelicolor

Martin Sanchez, Lara

Chapter 2

The γ-butyrolactone receptor protein ScbR is involved in the growth phase-dependent expression of the γ-butyrolactone synthetase ScbA in *Streptomyces coelicolor*

Lara Martin-Sanchez[1], Robert Bunet[1,2], Maria Elena Merlo[1], Lubbert Dijkhuizen[1], Mirjan Petrusma[1], Eriko Takano[1,3]

[1]Microbial Physiology, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, Nijenborgh 7, 9747AG Groningen, The Netherlands

[2]Present address: Équipe de Biologie Moléculaire Marine – PROTEE, Université du Sud Toulon-Var, BP 20132, Avenue de l'Université, 83957 La Garde Cedex, France

[3]Present address: Faculty of Life Sciences, Manchester Institute of Biotechnology, University of Manchester, 131 Princess Street, Manchester M1 7DN, United Kingdom

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Abstract

The γ-butyrolactone signalling molecules regulate antibiotic production in *Streptomyces coelicolor*. These diffusible signalling molecules are called SCBs (*S. coelicolor* butyrolactones) and are synthesised by ScbA. The SCB receptor protein ScbR is an autorepressor that binds to the promoter region of its own gene (site R) repressing its transcription. ScbR directly controls the expression of the CPK biosynthetic gene cluster by repressing expression of *cpkO*, encoding an activator of the cluster. Another binding site for ScbR exists in the promoter region of *scbA* (site A). In this work, site A was mutated (mutant LW145) resulting in a loss of ScbR binding. Mutant LW145 showed early *scbA* transcription resulting in early production of SCBs, detectable already in the exponential phase of growth, and strong ScbR expression from exponential phase onwards. Furthermore, mutant LW145 was affected in Act and Red production and showed a delay in production of the yellow pigment coelimycin P2, an amino acid adduct of an intermediate of the pathway for the synthesis of the antibiotic coelimycin A. Our data show that ScbR has a growth phase–dependent role in repressing expression of *scbA* and is also involved in controlling the induction of *scbA* transcription, probably aided by another protein.

Introduction

Streptomyces is a genus of Gram-positive soil-dwelling bacteria, with great relevance in medicine and biotechnology. Not only are these bacteria one of the main sources of natural antibiotics used in medicine nowadays, but they also have the genetic potential to provide additional novel antibiotics, judged from the richness in putative secondary metabolites cryptic gene clusters in their genomes (Omura *et al.*, 2001, Bentley *et al.*, 2002, Ikeda *et al.*, 2003, Mochizuki *et al.*, 2003, Ohnishi *et al.*, 2008, Medema *et al.*, 2010, Olano *et al.*, 2014). In *Streptomyces*, signalling molecules are involved in regulation of antibiotic production and morphological differentiation (van Wezel *et al.*, 2011, Willey *et al.*, 2011). These low molecular weight diffusible molecules called γ-butyrolactones act in nano-molar concentrations by binding to their receptor proteins that are usually transcriptional repressors.

Streptomyces coelicolor A3(2), the model organism of the genus, is known to produce at least 5 different antibiotics; the plasmid encoded methylenomycin (Wright *et al.*, 1976a), the blue polyketide antibiotic actinorhodin (Wright *et al.*, 1976b), the non-diffusible red pigmented undecylprodigiosin (Rudd *et al.*, 1980), the Calcium Dependent Antibiotic (CDA) (Hopwood *et al.*, 1983), and the recently discovered antibiotic coelimycin A (Gottelt *et al.*, 2010, Gomez-Escribano *et al.*, 2012, Challis, 2014). The γ-butyrolactones in *S. coelicolor* (SCBs, *S. coelicolor* butyrolactones) directly regulate the production of the antibiotic coelimycin A and the yellow pigments coelimycin P1 and P2 (Takano *et al.*, 2005, Takano, 2006, Gomez-Escribano *et al.*, 2012) and indirectly affect the production of actinorhodin (Act) and undecylprodigiosin (Red) (Takano *et al.*, 2000, Takano *et al.*, 2001). Three SCBs have been identified so far in this bacterium; SCB1 (the most abundant), SCB2 and SCB3. Their chemical structures have been elucidated (Takano *et al.*, 2000, Hsiao *et al.*, 2009b).

The *S. coelicolor* Butanolide (SCB) system has been studied in considerable detail. ScbA is the γ-butyrolactone synthetase (Hsiao *et al.*, 2007). ScbR is the protein responsible for the reception of SCBs (Takano *et al.*, 2001). Regulation of this system is achieved via a complex growth phase–dependent mechanism. The genes encoding for ScbA and ScbR lie adjacent and divergently oriented on the chromosome. ScbR is a transcriptional repressor and an autoregulator. In the exponential phase of growth, basal levels of ScbR are present, which are sufficient to bind to the DNA at site R, upstream of its own promoter region,

blocking its own transcription. Expression of *scbA* is activated in transition phase of growth by a yet unknown mechanism, resulting in the synthesis of SCBs by ScbA. The signalling molecules bind to their receptor protein ScbR and this provokes conformational changes of this protein that prevent its DNA binding ability. This results in activation of *scbR* transcription and accumulation of ScbR protein during transition phase.

An additional ScbR binding site (site A) was found in the promoter region of *scbA* by gel retardation assays (Takano *et al.*, 2001). DNAse I footprinting analyses showed that site A overlaps with the *scbA* core –10/–35 region. It was hypothesised that ScbR represses the expression of *scbA* by binding to site A. The exact mechanism by which *scbA* expression is then induced is still unknown, but it seems to involve the action of both ScbR and ScbA (Takano *et al.*, 2001).

The SCB system is involved in the *indirect* regulation of Act and Red synthesis in *S. coelicolor*: overproduction of Act and Red was observed in a *scbA* deletion mutant, while a *scbR* deletion mutant showed a delay in production of both antibiotics (Takano *et al.*, 2001). Yet, ScbR does not bind to the promoter regions of the pathway-specific regulators for Act and Red production (Takano *et al.*, 2001). ScbR *directly* regulates the expression of the CPK antibiotic biosynthetic gene cluster by repressing *cpkO*, encoding for a SARP (*Streptomyces* Antibiotic Regulatory Protein) activator of the cluster (Takano *et al.*, 2005). Two different ScbR binding sites have been found in the promoter region of *cpkO* by gel retardation assays; site OA, whose sequence shows high similarity to site R, and site OB, which resembles the site A sequence. As reported in Takano *et al*. (2005), ScbR binds to site OA, in the core promoter of *cpkO*, in exponential phase of growth repressing transcription of the gene (Takano *et al.*, 2005). When SCBs are synthesised in transition phase, they bind to ScbR, which dissociates from the DNA at this site, thus allowing transcription of the *cpkO* gene and, consequently, of the CPK cluster (Takano *et al.*, 2005). The CPK biosynthetic gene cluster is regulated by a complex mechanism not yet fully elucidated (Gottelt *et al.*, 2010). Besides ScbR and CpkO, at least two other proteins control the expression of the CPK biosynthetic gene cluster; the SARP activator CpkN, and the pseudo-γ-butyrolactone repressor protein ScbR2, whose genes are located in the CPK gene cluster (Pawlik *et al.*, 2007, Gottelt *et al.*, 2010, Xu *et al.*, 2010b). ScbR2 binds to the promoter region of *cpkO*

repressing the transcription of this gene in late transition phase which suggests a negative feedback mechanism to eventually reduce the expression of the CPK cluster in late stages of transition phase (Gottelt *et al.*, 2010, Xu *et al.*, 2010b).

In this study, the role of the SCB receptor protein ScbR in expression of *scbA* in *S. coelicolor* M145 was assessed by introducing four point mutations in the DNA sequence of site A, yielding mutant LW145. These mutations were introduced in such a way that the sequence of site A differed from the ScbR binding consensus sequence, which resulted in inability of ScbR to bind to this site as seen by gel retardation assays *in vitro*. Further characterisation of mutant LW145 revealed that ScbR is responsible for the growth phase–dependent repression of *scbA* expression but also is involved in induction of *scbA* expression, probably in combination with other proteins. The data presented here extend our understanding of the complex regulation of the SCB system and of the mechanism by which this system regulates antibiotic production in *S. coelicolor*.

Materials and methods

Bacterial strains, plasmids and growth conditions

Streptomyces coelicolor strains were manipulated as described previously (Kieser *et al.*, 2000). *Escherichia coli* strains JM101 and ET12567 were handled according to standard procedures (Sambrook *et al.*, 1989). MS agar (Kieser *et al.*, 2000) was used to make spore suspensions and for plating out conjugations, and to observe morphological differentiation. R2, R2YE, SMMS (Kieser *et al.*, 2000), modified SMMS (MSMMS; without casamino acids and supplemented with 325 mM glutamate) (Gottelt *et al.*, 2010) and a 2% agar version of the minimal medium described in Nieselt *et al.* (2010), were used to assess antibiotic production of *S. coelicolor* strains in solid media. Table S1 lists the plasmids used in this work.

Construction of a site A mutated strain of *S. coelicolor*

In order to alter the sequence of site A to differ from the ScbR binding consensus sequence, four point mutations were introduced in this site in the wild type strain *S. coelicolor* M145. To do this, the *scbA*/*scbR* region was amplified by PCR with RB5F and RB5R primers (all primers used in this work are listed in Table S2) using SCAH10 as template (Redenbach *et al.*, 1996) to yield a 1930 bp fragment. This fragment was cloned into pGEM-T Easy vector (Promega) to obtain the plasmid pTE74. The sequence of the amplified 1.9 kb fragment in pTE74 was verified by sequencing. The sequence of the ScbR-binding site A was altered using a modified version of the site-directed ligase-independent PCR-mediated mutagenesis (SLIM) method (Chiu *et al.*, 2004). A 92 nt oligonucleotide, BindingA, containing the point mutations, was amplified by PCR using primers BindA-fwd and BindA-rev. 100 ng of the 92 bp fragment were used in combination with flanking primers, SLIM1 (10 pM) and SLIM2 (10 pM), to amplify pTE74 by inverse PCR using a mixture of Taq DNA polymerase (5 units, Qiagen) and ProofStart DNA polymerase (0.2 unit, Qiagen). PCR products were purified with the Qiagen PCR purification kit and pTE74 was removed by treatment with 10 units of *Dpn*I (New England Biolabs). The conditions for SLIM heteroduplex formation were performed as described in (Chiu *et al.*, 2004). 5 µl of reaction mixture were used to transform competent *E. coli* JM101 cells. Clones were screened by colony PCR for the desired mutations using the detection primer, VerifA-fwd, which contains at

the 3' end, the GCC triplet (instead of CGG present in the wild type binding site A). This primer in combination with primer scbRrt1 allowed amplification of a PCR fragment only with the plasmid containing the modified binding site A as template. The resulting plasmid was named pTE75. The *Eco*RI 1.9 kb fragment from pTE75 was subcloned into the *Eco*RI site of the conjugative vector pKC1132 (Bierman *et al.*, 1992), to yield pTE76. Sequencing of this plasmid revealed that it contained an additional undesired point mutation in the *scbA* coding region. This mutation was then corrected by re-amplification of this plasmid with primers Fix-scbApm_pTE76_F and Fix-scbApm_pTE76_R containing the wild type corrected base according to the QuikChange mutagenesis method (Agilent Technologies). The obtained modified plasmid (pTE76*) contained the desired four mutations in site A, and no other additional mutations, as confirmed by sequencing. The *scbA/scbR* region including the mutated site A was cloned in pTE33.

pTE33 is a derivative of the *E. coli/Streptomyces* shuttle vector pKC1132 (Bierman *et al.*, 1992), and contains a 7.5 kb DNA fragment that includes genes *sco6263* to *sco6269* flanking the *scbA/scbR* region. The 7.5 kb fragment in this plasmid was obtained from two other plasmids, pIJ6111, harbouring a 4.5 kb DNA fragment including genes from *sco6263* until approximately the 5' end of *sco6266* (*scbA*), and pIJ6114, harbouring the adjacent 3 kb DNA fragment including the end of *scbA* and genes sco6267-sco6269 (Takano *et al.*, 2001). First, pIJ6114 was digested with *Bam*HI and *Xmn*I to yield a fragment of 3 kb. This *Bam*HI-*Xmn*I DNA fragment was ligated to pCR-BluntII-TOPO digested with *Bam*HI and *Eco*RV, and the resulting vector was called pTE31. pIJ6111 was then digested with *Bam*HI, and the 4.5 kb DNA fragment obtained was isolated and ligated to pTE31 digested with *Bam*HI. Plasmids with the 4.5 kb fragment inserted in the right orientation were screened by restriction digestion profiling. The resulting plasmid (pTE32) contains the 7.5 kb fragment containing *scbA* and *scbR*. pTE32 was digested with *Hin*dIII and *Xba*I to isolate the 7.5 kb fragment and it was ligated to pKC1132, a suicide vector for conjugation between *E. coli* and *Streptomyces*, to yield pTE33. The complete 7.5 kb DNA fragment included in this plasmid was sequenced to confirm the absence of mutations.

A 1.2 kb *Pst*I-*Nru*I DNA fragment, including most of *scbA* and *scbR* from pTE76*, was ligated into pTE33 to obtain pTE34. pTE34 was used to transform

the methylation deficient strain *E. coli* ET12567 carrying the non-transmissible mobilizing plasmid pUZ8002 (Paget *et al.*, 1999) and transferred by conjugation into *S. coelicolor* M145. Single-crossover exconjugants were selected on SFM containing apramycin and then taken through two rounds of non-selective growth on SFM to allow the double cross-over event that will lead to the loss of the plasmid and the apramycin resistance, and therefore to the allelic exchange. Apramycin-sensitive colonies were then selected and checked by PCR for the presence of the mutations in site A, using the primers VerifA-fwd and RCseq25. Positive clones were then sequenced to ensure the presence of the mutations in site A and the absence of additional mutations. The resulting mutant strain was called LW145.

Complementation of strain LW145

Complementation was performed by replacing the mutated site A by the wild type site A in *S. coelicolor* LW145. pTE33 was introduced into the methylation deficient *E. coli* strain ET12567 and transferred to *S. coelicolor* LW145 by conjugation. Insertion of the plasmid in the chromosome of LW145 was confirmed by selection with apramycin and by PCR. Double cross-over exconjugants were obtained after two rounds of non-selective growth in MS medium without antibiotics as described in the previous section. Positive clones were verified by PCR and by sequencing, confirming the absence of the four point mutations in site A.

Time-series experiments

S. coelicolor M145 (wild type), LW145 (site A mutant), M751 (Δ*scbA*) and M752 (Δ*scbR*) were grown in 50 ml of SMM liquid medium (3.45 x 10^8 spores) and incubated at 30°C at 220 rpm. Optical density at 450 nm (OD_{450}) was measured every hour from 16 to 26 h and at 42 h of incubation. Samples for RNA, protein, γ-butyrolactones (SCBs) and antibiotic isolation were collected at OD_{450} values corresponding to different phases of growth. These experiments were performed in triplicate to assess the reproducibility of the results.

Gel retardation assays

DIG-labelled DNA probes were made as follows. Using two combinations of primers ETS2/ETS4 or ETS4/ETS10 (see Table S2) on pTE74 (original site A) or pTE75 (mutated site), four DNA fragments were amplified by PCR, gel purified

and 1.5 µg of each PCR fragment were DIG-labelled with terminal transferase according to the instructions of the manufacturer (Roche, Dig Gel shift kit). Each gel retardation assay was conducted as in Takano *et al*. (2001) with 3.75 ng of DIG-labelled probes and 100 ng of pure ScbR (in a final volume of 20 µl).

Antibiotic production measurements

The antibiotic production of LW145 was studied in duplicate, in both liquid and solid media in comparison with M145, M751 and M752. In liquid media, Act and Red were extracted from SMM liquid cultures (samples were collected at the time points indicated in Figure 2) according to the procedure described by Strauch *et al*. (1991). To assess the antibiotic production of these strains in solid media, 1 x 10^8 spores of each strain were streaked out on R2, SMMS, and modified SMMS (without casaminoacids and with 325 mM L-glutamic acid monosodium salt (Gottelt *et al.*, 2010)).

Reverse transcription and quantitative RT-PCR

Gene expression studies were performed in the LW145 mutant strain by quantitative RT-PCR using random-primed cDNA in comparison with the expression profiles of the wild type strain M145, and with the *scbA* and *scbR* deletion mutants M751 and M752 (analyses were performed in two biological replicates of all the strains). RNA was isolated as described in Kieser, *et al.* (2000). For each sample, 10 µg of RNA were treated with 10 U of DNAseI (Roche) to remove the remaining DNA. RNA integrity and lack of DNA was assessed by electrophoresis in an agarose gel and by PCR, and its concentration measured using a nanodrop spectrophotometer (Thermo Scientific). RNA (2 µg) was used to synthesize random-primed cDNA as previously described (Gottelt *et al.*, 2010). qRT-PCR reactions were run on a BioRad CFX96 Real Time PCR Detection System in a reaction volume of 12 µl, using IQ-SYBR-Green Supermix (BioRad), 33.33 ng of cDNA and 5 pmol of each primer. The PCR reaction conditions were as follows: denaturation at 95°C for 3 min, then 40 cycles of denaturation at 95°C for 10 s and annealing at 60°C for 30 s, followed by a melt curve that includes a step at 95°C for 10 s and a 0.5°C increasing temperature gradient from 65° to 95° in intervals of 5 s. The *hrdB* gene, encoding for the RNA polymerase main sigma factor, was used as reference gene and the expression data for all the samples was normalised to

its mRNA levels. Relative quantification was achieved by comparing every normalised expression level to the expression level of the reference sample (TP1, exponential phase). All samples were run in triplicate. Data analysis was performed using BioRad CFX Manager 2.0. Primers used for these experiments (Table S2) amplified a cDNA fragment spanning the UTR region of *scbA* and *scbR* respectively, not in the coding region, so that the same primers could be used for the *scbA* and *scbR* deletion mutants (in which most but not all of the coding region was removed) (Takano *et al.*, 2001).

Protein separation and Western analyses

Protein was isolated from approximately 25 ml of SMM liquid cultures. Cells were vacuum-filtered, washed and disrupted by sonication (10 cycles, 15 sec on, 45 sec off) and with a French press (four cycles, 1,000 psi). Cell-free extracts were obtained by centrifugation and proteins were separated by SDS-PAGE (15% w/v SDS-polyacrylamide gels) according to Laemmli's procedure (Laemmli, 1970). Proteins were then transferred to a polyvinylidene (PVDF) membrane and treated with ScbR antibodies (Gottelt *et al.*, 2012) (dilution 1:10,000) as previously described (D'Alia *et al.*, 2010). This experiment was performed in two biological replicates.

Kanamycin bioassay

SCBs production in the various strains was assessed by means of the kanamycin bioassay (Hsiao *et al.*, 2009b). This bioassay makes use of a reporter strain, LW16, a *scbA* and *scbR* double deletion mutant, carrying the plasmid pTE134. This plasmid harbours a kanamycin resistance gene under the control of the *cpkO* promoter, which is directly repressed by ScbR, also encoded in the plasmid. If γ-butyrolactones are present in the medium, the repressive effect of ScbR on the *cpkO* promoter is relieved and the kanamycin resistance gene is expressed (Hsiao *et al.*, 2009b). The SCBs were extracted with 2 volumes of ethyl acetate from 50 ml SMM liquid culture supernatants. These extracts were dried, resuspended in 25 µl of methanol, and 5 µl were spotted in DNA agar plates containing 4.5 µg/ml kanamycin inoculated with the reporter strain LW16/pTE134 (2.6×10^6 spores). The plates were incubated at 30°C for 2 days. For a detailed protocol see Hsiao *et al*, 2009a. The results shown were obtained from three different biological replicates.

Extracts were also obtained from agar plates. Confluent lawns of the different strains were grown in SMMS agar plates (10 square plates, 12x12 cm) and incubated for 3 days at 30°C. SCBs were isolated from the agar with ethyl acetate and the extracts were dried and resuspended in 100 µl of 100% methanol as described in Takano *et al*. (2001).

Analyses of SCBs by LC/MS

Ethyl acetate extracts from the wild type M145, LW145, M751 (Δ*scbA*) and M752 (Δ*scbR*) grown in SMMS, and extracts from the time series experiment from wild type M145 and mutant LW145 were dried and resuspended in 100 % methanol. These extracts were then analysed using liquid chromatography (LC) on-line coupled to an Exactive Orbitrap-MS (Thermo Scientific Corporation). Chemically synthesised racemic SCB1 and SCB2 (100 ng/µl) (Chapter 3), were used as standards, and strain Δ*scbA* ethyl acetate extracts were injected as negative control. The LC/MS system was operated with the electrospray ionization source in positive mode. LC conditions were as follows: the column was a Reversed Phase C18 column (Shim-pack XR-ODS, Shimadzu, 3 µm, 75 µm i.d. x 15 cm); the mobile phases consisted of A (0.1% (v/v) Formic Acid in Water) and B (0.1% (v/v) Formic Acid in Acetonitrile). The gradient elution (24 min in total) started with 30% B for 1 min, 50% B at 22 min, 50% B hold for 1 min and back to initial phase 30% B at 24 min; flow rate 0.6 µl/min; MS mass detection range 80-800 Da. All solvents were LC-MS grade (Biosolve). 1 µl of the standards and 5 µl of the ethyl acetate extracts were injected. 3 technical replicates were run for each sample. Data analysis was done using the Xcalibur software (Thermo Fisher Scientific).

Results

Construction of site A mutant strain LW145 and complementation

To assess the effects of the ScbR protein on the expression of the *scbA* gene, the ScbR binding site A (located in the promoter region of *scbA*) was mutated on the chromosome of the wild type strain *S. coelicolor* M145. As shown in Figure 1, four point mutations were inserted in site A that modified it from the ScbR binding consensus sequence. These mutations were introduced outside of the –10/–35 region, which resembles the consensus recognition sequence for the major sigma factor HrdB of *S. coelicolor* (Brown *et al.*, 1992, Aigle *et al.*, 2000) and was kept unmodified. The resulting mutant strain was obtained by replacement of the original site A by the mutated site A by double cross-over recombination using plasmid pTE34 (Table S1); it was named LW145. The sequence of the *scbA–scbR* region was verified by sequencing.

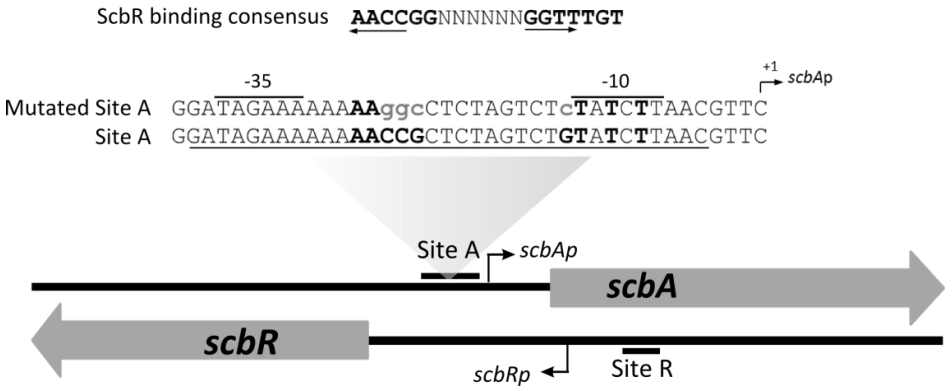


Figure 1. Mutations in site A, the ScbR binding site in the *scbA* promoter region. The four point mutations in site A (in grey lower case) that modified it from the ScbR binding consensus sequence without affecting the –10/–35 region. Conserved nucleotides from the ScbR consensus sequence are shown in bold. Numbers (–10) and (–35) are with respect to the *scbA* transcriptional start site. The transcriptional start sites of *scbA* and *scbR* are denoted by bent arrows (*scbA*p, *scbR*p). The ScbR binding consensus sequence is indicated above. Inverted repeats are indicated by arrows.

The phenotype of the site A mutant LW145 was studied in different phases of growth, in comparison with the wild type strain M145 and the mutants M751 (Δ*scbA*) and M752 (Δ*scbR*). The strains were grown in SMM liquid medium, and samples were taken at different time points during growth. Samples were used

to isolate RNA, protein, antibiotics and γ-butyrolactones (SCBs). The time points at which samples were collected and the corresponding phase of growth are indicated in Figure 2. All mutated strains were similar in growth compared to the wild type strain M145.

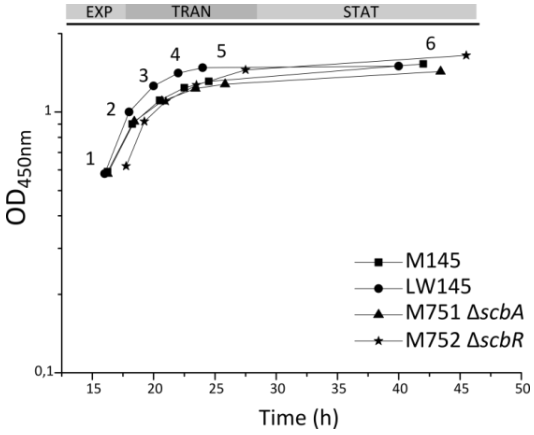


Figure 2. Growth curves of wild type strain M145, and mutant strains LW145, M751 and M752 in SMM medium. Samples were collected at the time points indicated on top of the curves (1 to 6). The phase of growth that corresponds to each time point is indicated at the top as EXP, exponential phase, TRAN, transition phase, and STAT, stationary phase.

A complementation mutant was constructed by replacing the mutated site A by a wild type copy by double cross-over recombination. Plasmid pTE33 was transferred into strain LW145 by conjugation and the double cross-over recombinants were selected and confirmed by sequencing. The phenotype of this complementation mutant was restored to that of the wild type.

ScbR cannot bind to the mutated site A *in vitro*

To determine whether ScbR could bind to the mutated site A *in vitro*, gel retardation assays were performed with DNA fragments containing either the original site A or the mutated site A (site A*). The relative positions of the fragments used for these experiments are shown in Figure 3, A.

When the original site A was tested, retarded DNA-ScbR complexes were detected. No retardation was detected when the DNA fragment containing the mutated site A was used (Figure 3, B). Furthermore, when a fragment

containing both mutated site A and site R was used (fragment A*-R), only two retarded complexes were detected, as opposed to the four complexes detected when using a DNA fragment including the original site A and site R (fragment A-R), indicating that ScbR is only binding to site R in this DNA fragment (Figure 3, B).

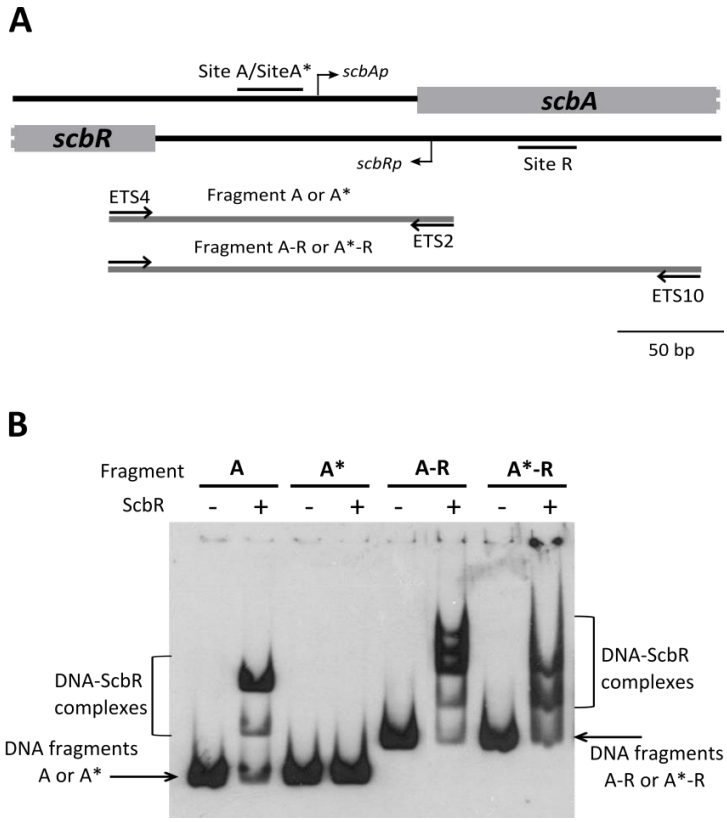


Figure 3. Inability of the ScbR protein to bind to the mutated site A. *A*, DNA fragments used for the assay were amplified by PCR using primers ETS4, ETS2 and ETS10, indicated with arrows. Fragment A and A* contain only the original site A or mutated site A, respectively. Fragments A-R and A*-R include also site R. *scbA*p and *scbR*p, depicted by bended arrows, denote the transcriptional start site of *scbA* and *scbR* respectively (Takano *et al.*, 2001). *B*, Gel retardation assays using DIG-labelled DNA fragments containing either original site A (fragment A) or mutated site A (fragment A*) and either the original site A and site R (fragment A-R) or mutated site A and the original site R (fragment A*-R) with (+, 100 ng) or without (–) pure ScbR.

Transcription levels of *scbA* and *scbR* are altered in the site A mutant LW145

To assess the effects of the mutation in site A on the transcription of *scbA* and *scbR*, qRT-PCR experiments were performed. RNA was isolated from samples collected at different phases of growth of M145, LW145, M751 and M752 grown in SMM medium (see Figure 2). The results shown were reproducible in two biological replicates.

As shown in Figure 4, levels of *scbA* transcripts increased dramatically in the early and mid-transition phase in wild type strain M145, and fell quickly in late transition phase. Compared to wild type, the site A mutant LW145 showed increased levels of *scbA* transcript in exponential phase. However, no induction of *scbA* expression was observed in transition phase, at the same time point at which it was observed for the wild type. It is possible that the *scbA* induction peak was slightly shifted in time; however, we did not detect any increase in *scbA* transcription at an intermediate time point between time point 3 and 4 (data not shown). A similar expression profile was observed in the *scbR* deletion mutant, with higher expression levels for *scbA* in exponential phase compared to the wild type and loss of induction in transition phase, although the overall amount of *scbA* transcript detected was lower compared to the amounts detected in the site A mutant. These results suggest that ScbR is involved in controlling the induction of *scbA* transcription.

Only basal levels of *scbA* transcripts were detected for the *scbA* deletion mutant M751, assessed by amplifying a region in the 5' untranslated region (UTR) of this gene, which is still present in the deletion mutant.

The expression of *scbR* increased gradually in time in M145 until it reached its maximum in mid-transition phase to start decreasing again gradually until stationary phase (Figure 4). Levels of *scbR* transcript in Δ*scbA* were strongly reduced compared to the wild type, while *scbR* was overexpressed in Δ*scbR* (Figure 4), as determined by amplifying a region in the 5' UTR of the *scbR* gene, as was previously reported (Takano *et al.*, 2001). *scbR* was also overexpressed in mutant LW145, from the earliest time point in exponential phase onwards, resembling the expression profile obtained for Δ*scbR*. Thus, there is no repressive effect of ScbR on its own transcription in mutant LW145.

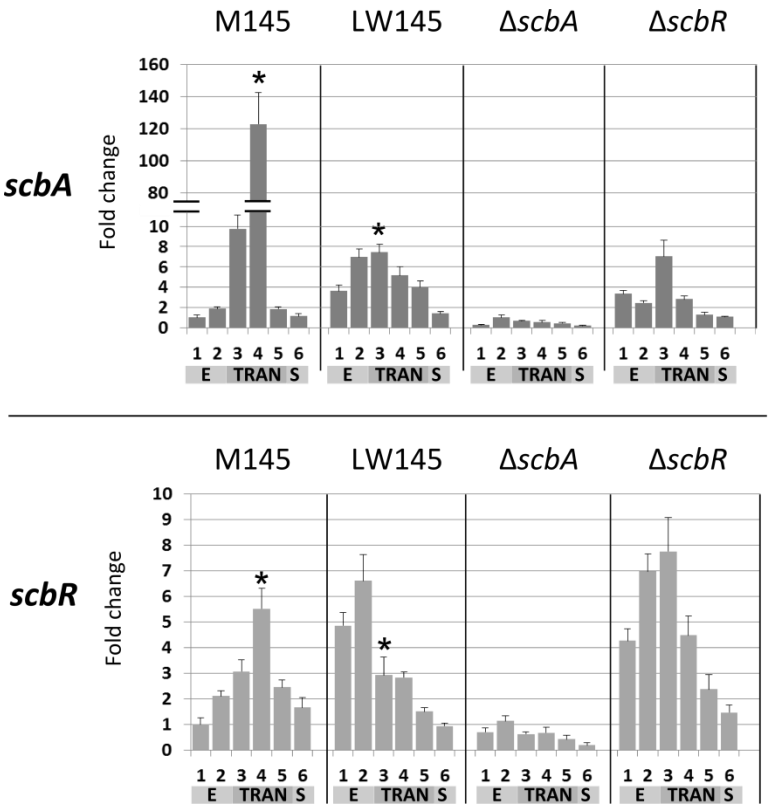


Figure 4. qRT-PCR analysis of *scbA* and *scbR* expression along growth of *S. coelicolor* strains. Expression levels are shown as fold change relative to the levels of the M145 sample at time point 1 (exponential phase). Numbers indicate the time points at which RNA samples were collected (see Figure 2) and its correspondence with the growth phase is indicated below (E, exponential, TRAN, transition, S, stationary phase). Asterisks indicate the onset of γ-butyrolactone production in M145 and LW145, as detected with the kanamycin assay.

Expression of ScbR is constitutive in the site A mutant strain LW145

ScbR is an autoregulator that represses its own transcription. *scbR* was overexpressed in the site A mutant LW145, in similar levels as in the *scbR* deletion mutant M752. To assess whether the high levels of *scbR* transcript actually correspond to high levels of ScbR protein in this mutant, Western Blot analyses were performed using ScbR antibodies. Protein samples were isolated from M145, LW145, Δ*scbA* and Δ*scbR* cultures grown in SMM medium, collected at different stages of growth, at the same time points at which the RNA samples used for the qRT-PCR experiments were collected (Figure 2).

Levels of ScbR protein increased in M145 as the culture progressed into transition phase to reach a maximum level in stationary phase (Figure 5). In contrast, ScbR protein was detected in higher levels already from exponential phase onwards in LW145, and these high levels further increased as the culture entered stationary phase. No ScbR protein was detected in the *scbA* deletion mutant. As expected, the ScbR protein was also absent from the *scbR* deletion mutant M752 (Figure 5).

The constitutive expression of ScbR from exponential phase onwards in LW145 agrees with the overexpression of the *scbR* gene detected by qRT-PCR in this phase of growth. This suggest that ScbR is no longer repressing its own gene expression in this mutant. This in turn suggests that ScbR is not binding to site R *in vivo* in the conditions examined, despite the fact that binding of ScbR to site R is observed in the gel retardation assays *in vitro* and although the sequence of site R was not modified in the site A mutant LW145 as checked by sequencing.

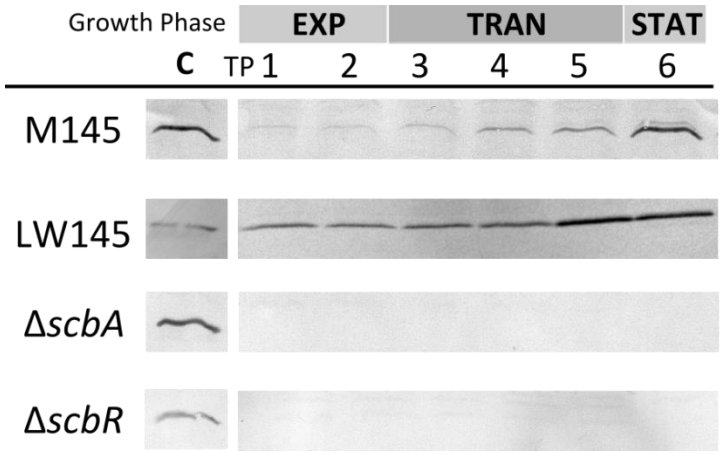


Figure 5. Western blot analysis for the detection of the ScbR protein during growth of *S. coelicolor* strains. Protein samples were obtained from liquid cultures of M145, LW145, M751 Δ*scbA* and M752 Δ*scbR* at different time points of growth. Western blots were performed using ScbR antibodies. Numbers indicate the time points (TP) at which the samples were collected (see Figure 2). EXP, TRAN and STAT indicate the phase of growth corresponding to every time point (EXP, exponential, TRAN, transition, STAT, stationary phase). C, control sample containing ScbR protein.

LW145 shows an early production of SCBs

To further analyse the *in vivo* effects of the inability of ScbR to bind to the mutated site A, the SCBs production profile along growth in liquid medium was studied in the site A mutant LW145 by means of the kanamycin assay (Hsiao *et al.*, 2009b), and compared to the profiles of the wild type strain M145 and the *scbA* (M751) and *scbR* (M752) deletion mutants. Samples were collected at the time points indicated in Figure 2.

Only wild type strain M145 extracts from mid-transition to stationary phase of growth activated the growth of the reporter strain (Figure 6, A). The onset of SCBs production in M145 thus occurs in mid-transition phase, as previously reported (Takano *et al.*, 2001), at the same time point at which the peak in *scbA* transcription was detected by qRT-PCR. In LW145, the site A mutant strain, SCBs are produced earlier, and are already detectable in early transition phase (Figure 6, A). These results were reproducible for three different biological replicates. The early production was also confirmed by LC/MS analyses. The same samples used for the kanamycin assays were run in duplicate in the LC/MS and their chromatograms and mass spectra compared to the chemically synthesised SCB1 and SCB2 standards (Chapter 3). The SCBs were detected already in late exponential phase in LW145 extracts, in contrast to the wild type extracts, where signalling molecules were only detected in early transition phase (Figure 7). No SCBs were detected in time point 1, corresponding to early exponential phase in the site A mutant LW145.

The size of the halo of growth detected with the kanamycin bioassay depends on the amount of γ-butyrolactones present; in general terms, the higher the amount of γ-butyrolactones present, the bigger the diameter of the halo (Hsiao *et al.*, 2009b). Our results show that the site A mutant LW145 is producing reduced amounts of SCBs compared to the wild type strain, since the diameter of the halo of growth observed with the LW145 extracts is slightly smaller than the one observed with M145 extracts (Figure 6). This pattern was also confirmed by LC/MS analyses. SCB1 was detected in the LW145 strain in amounts 3 times lower than in the wild type, and amounts of SCB2 detected were 5 times lower (Figure 8).

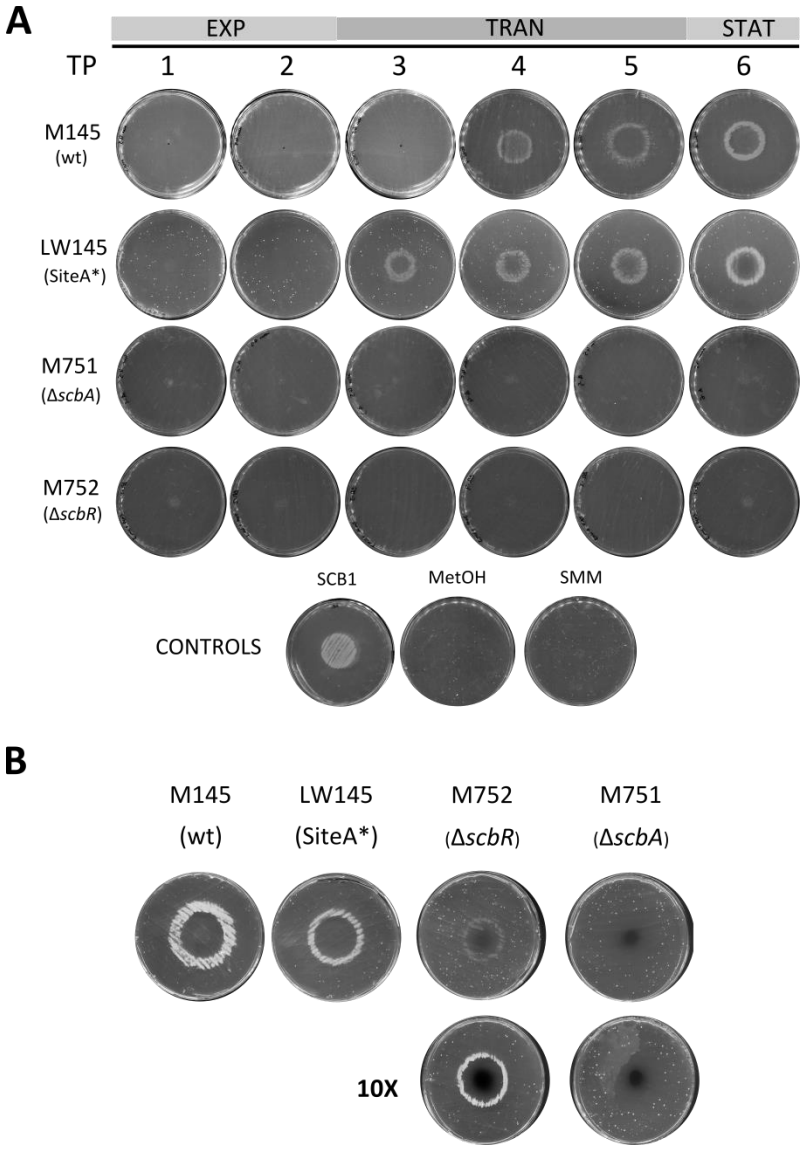


Figure 6. Kanamycin assay for the detection of γ-butyrolactones along growth of *S. coelicolor* strains. A, Ethyl acetate extracts obtained from liquid culture supernatants of M145 (wild type strain), LW145 (site A mutant), and the *scbA* and *scbR* deletion mutants, collected at exponential (EXP), transition (TRAN) and stationary (STAT) phase, were tested. A halo of growth of the reporter strain indicates the presence of γ-butyrolactones in the extracts. Controls: 0.4 µg of chemically synthesised racemic SCB1, Methanol and an ethyl acetate extract of the SMM medium used for the liquid cultivations. B, Extracts were obtained from confluent lawns of the different strains growing on SMMS agar plates. A Δ*scbR* extract concentrated 10 times (indicated as

10X), succeeded in activating the growth of the reporter strain, while a Δ*scbA* extract did not. The diameter of the halo observed for Δ*scbR* was considerably reduced compared to non-concentrated M145 extracts (on the top).

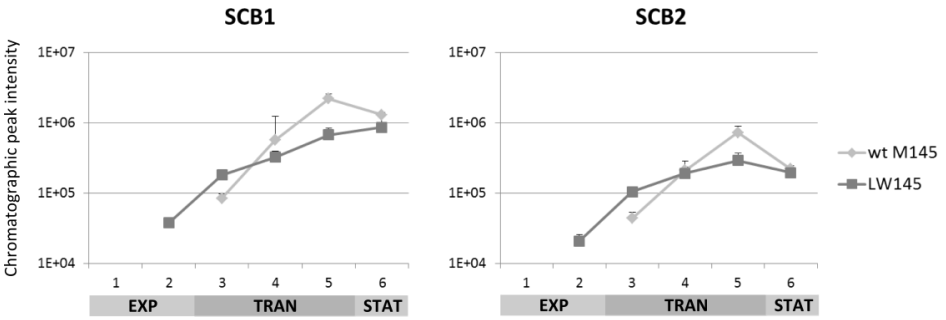
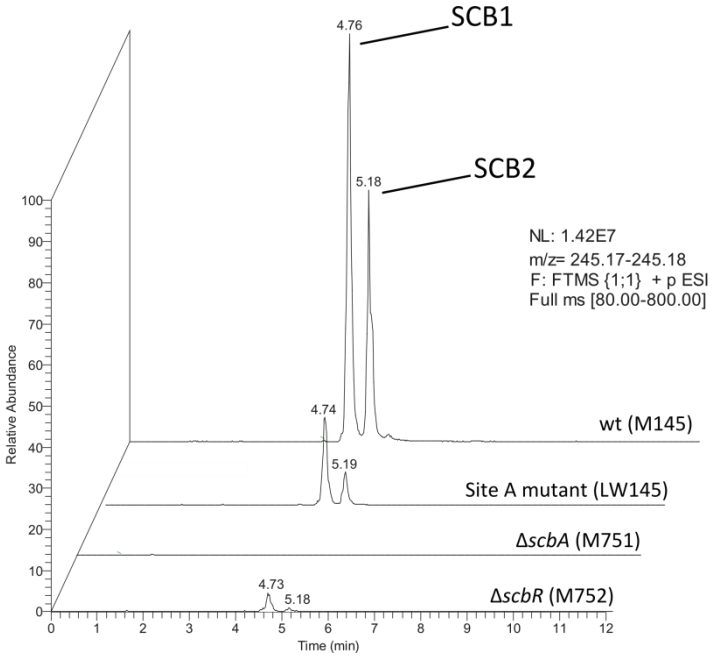


Figure 7. Relative amounts of SCB1 and SCB2 produced in SMM liquid medium during growth of *S. coelicolor* strains in liquid medium, as detected by LC-MS. The graphs are showing the absolute intensities of the chromatographic peaks (mass range 245.1740-245.1760 Da) in logarithmic scale, obtained for SCB1 and SCB2 by LC-MS, at different time points in LW145 compared to M145. The chromatographic peaks and mass spectra of SCB1 and SCB2 in the time series samples match with peaks and mass spectra obtained from LC-MS analysis of the chemically synthesised standards SCB1 and SCB2 confirming the identification of these compounds (as shown in Figure S1). The values shown are the average of three technical replicates. The standard deviation is indicated with error bars.

No growth of the reporter strain was detected when using the M751 (Δ*scbA* mutant) and M752 (Δ*scbR* mutant) extracts, in any phase of growth, showing that no detectable amounts of SCBs are present in these samples (Figure 6, A). Similar results were previously reported in Takano *et al*. (2001) where the standard bioassay for γ-butyrolactones, (that tests the ability of the extracts to induce precocious Act and Red in a lawn of M145) which shares the same detection sensitivity toward SCB1, was used (Hsiao *et al.*, 2009b). However, when a 10 times concentrated M752 extract was tested with the kanamycin bioassay, growth of the reporter strain was detected, suggesting that the *scbR* deletion mutant strain does produce SCBs but in lower amounts than the wild type strain (Figure 6, B). The reporter strain did not show any growth when 10 times higher concentration of extracts of M751 (*scbA* deletion mutant) were used (Figure 6, B).

The results of the Kanamycin bioassays for the *scbR* deletion mutant were confirmed by high-resolution LC/MS mass spectrometry of ethyl acetate

extracts obtained from cultures on SMMS solid medium. The chromatographic peaks and their corresponding mass spectra detected in the ethyl acetate extracts of M145 and M752 (Δ*scbR*) matched with the peaks and masses detected with the chemically synthesised SCB1 and SCB2 (Figure S1). The intensity of the peaks obtained for the *scbR* deletion mutant was however notably low, about 15 times lower than the intensity of the peak obtained for the wild type strain for SCB1 and about 60 times lower for SCB2 (Figure 8). The results shown in Figure 8 correspond to one biological replicate. Ethyl acetate extracts from an independent experiment used as a second biological replicate were also analysed by LC/MS and confirmed the strong reduction in SCB1 and SCB2 levels detected in M752. These results confirm that the *scbR* deletion mutant has the ability to produce SCBs although in considerably lower amounts than the wild type strain M145.



	SCB1	Fold change	SCB2	Fold Change
M145	$1.11x10^7$		$7.41x10^6$	
LW145	$3.39x10^6$	3.28x	$1.31x10^6$	5.64x
M752	$7.2x10^5$	15.5x	$1.17x10^5$	63.3x
M751	N.D.		N.D.	

Figure 8. LC-MS detection of SCB1 and SCB2 in extracts from cultures on SMMS solid media of M145, LW145, Δ*scbA* (M751) and Δ*scbR* (M752). SCB1 and SCB2 are detected in the extracts of M145, LW145 and Δ*scbR* (M752), while no corresponding peaks are detected in the mutant strain Δ*scbA* (M751). The LC-MS data for SCB1 and SCB2 in these biological extracts match in retention time and mass spectra with the detections obtained with chemically synthesised standards (Figure S1), confirming the identity of the molecules. The table on the right shows the average intensity values of SCB1 and SCB2 chromatographic peaks from three technical replicates. The difference in amounts detected in the LW145 and M752 is shown as fold change compared to the intensities obtained for the wild type M145. N.D., not detected. Mass Spectra are shown in the supplemental material (Figure S1).

Production of coelimycin P2 is delayed in LW145 and in the *scbR* deletion mutant, and absent in the *scbA* deletion mutant

ScbR directly regulates the expression of the CPK biosynthetic gene cluster by repressing the transcription of *cpkO*, encoding CpkO, an activator of the CPK cluster. The SCBs activate the expression of *cpkO* by avoiding ScbR from binding to its promoter which results in activation of expression of the gene (Takano *et al.*, 2005). To evaluate whether the early production of SCBs detected in LW145 affects the production of the CPK metabolites, the ability of this mutant strain to produce coelimycin P2, a yellow glutamate adduct the antibiotic coelimycin A (Gomez-Escribano *et al.*, 2012, Kotowska *et al.*, 2014), was assessed. Wild type M145, mutants LW145, M751, M752, LW16 (*scbA*/*scbR* double deletion mutant) and the LW145 complementation mutant (cLW145) were grown on agar media supplemented with glutamate (MSMMS medium), and pigment production was compared.

A yellow coloration was observed for M145 (and cLW145) already 27 h after inoculation, while it was clearly delayed in the site A mutant LW145 (Figure 9). Production of coelimycin P2 was not detected for the *scbA* deletion mutant M751, as previously reported (Gottelt *et al.*, 2010), while the Δ*scbR* mutant M752 showed a delayed and reduced production of the pigment. Coelimycin P2 production in the *scbA/scbR* double deletion mutant LW16 resembles the production profile observed for the LW145 mutant (Figure 9).

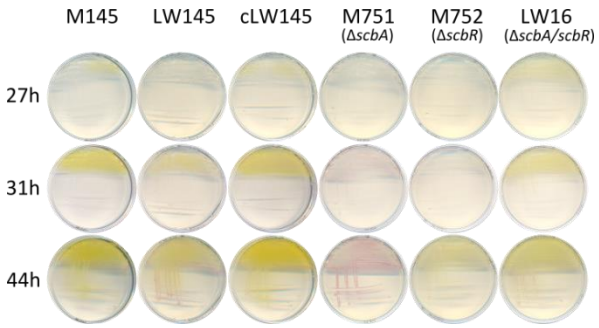


Figure 9. Production of coelimycin P2 in LW145 compared to wild type M145, M751 (Δ*scbA*), M752 (Δ*scbR*), LW16 (*scbA/scbR* double deletion mutant), and the LW145 complementation mutant (cLW145). Spores of the different *S. coelicolor* strains were streaked out on MSMMS (modified SMMS medium) agar, optimised for coelimycin P2 production, visualised as yellow coloration. The pink coloration corresponds to Red production.

Expression of *cpkO* and *scbR2* is reduced but starts earlier in LW145 in comparison to the wild type

SCBs activate the expression of *cpkO,* encoding the SARP activator of the CPK cluster, by binding to the repressor ScbR. To assess the effects of the early onset of SCBs on transcription levels of *cpkO* and *scbR2*, regulators of the CPK cluster, qRT-PCR experiments were performed in the LW145 mutant and compared to M145.

In the site A mutant, levels of *cpkO* expression increased in early transition phase in comparison with the wild type M145 (Figure 10). However, the expression levels of this gene were greatly reduced in mid-transition phase compared to M145. Expression of *scbR2* in M145 occurred after the increase in *cpkO* transcript levels, in late-transition phase. Interestingly, transcript levels of *scbR2* were detected earlier (early transition phase) and in higher amounts in the site A mutant (Figure 10).

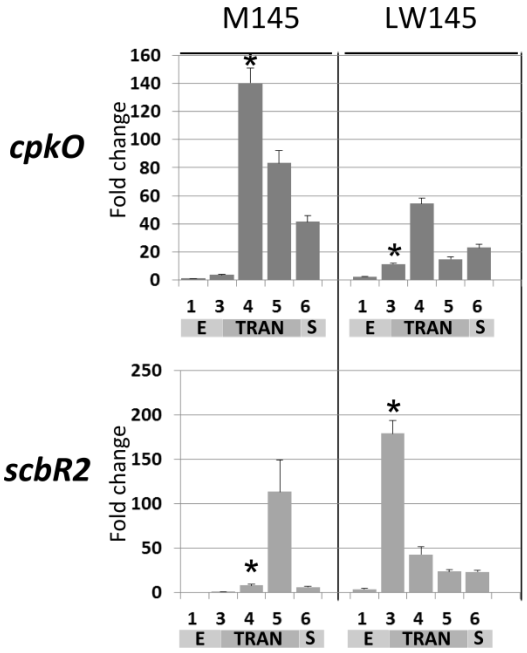


Figure 10. qRT-PCR analysis of *cpkO* and *scbR2* expression along growth of *S. coelicolor* strains. All levels are normalised to that of *hrdB*. Relative quantification of the transcription levels is shown as fold change relative to the levels for M145 sample at time point 1. Asterisks indicate the onset of γ-butyrolactone production in the M145 and LW145 strains.

Act and Red are produced earlier in the site A mutant LW145 compared to the wild type

The SCB system has been suggested to be involved in regulation of production of the antibiotics Act and Red in an indirect manner (Takano *et al.*, 2000, Takano *et al.*, 2001). To elucidate the effects of the mutation in site A on Act and Red production, the levels of these compounds were analysed in solid and liquid media in the site A mutant LW145 compared to the wild type and *scbA* and *scbR* deletion mutant strains.

An earlier onset of Red production was observed in the site A mutant LW145 compared to the wild type strain M145 when grown on SMMS solid medium (Figure 11, A). As previously reported, the *scbA* deletion mutant showed an overproduction of Act and Red compared to M145 while in the *scbR* deletion mutant the production of Red was delayed (Takano *et al.*, 2001). It is noteworthy that no differences in Act and Red production were observed for the *scbA/scbR* double deletion mutant LW16 compared to the wild type.

Act and Red production in liquid SMM medium was also assessed using samples at the time points indicated in Figure 2. The spectrophotometric quantification showed that LW145 mostly produced Act and Red in similar amounts as the wild type strain M145 (Figure 12). However, very low levels of Act were already reproducibly detected in early transition phase in LW145, while production started in mid-transition phase in the wild type (Figure 12, B). These results show that the onset of Act production in LW145 occurs earlier than in the wild type, in early transition instead of mid-transition phase, in liquid media. As seen also in solid medium, the Δ*scbA* strain overproduced Act and Red, and the production was delayed and considerably reduced in Δ*scbR* compared to both the site A mutant LW145 and the wild type (Figure 12, A, B).

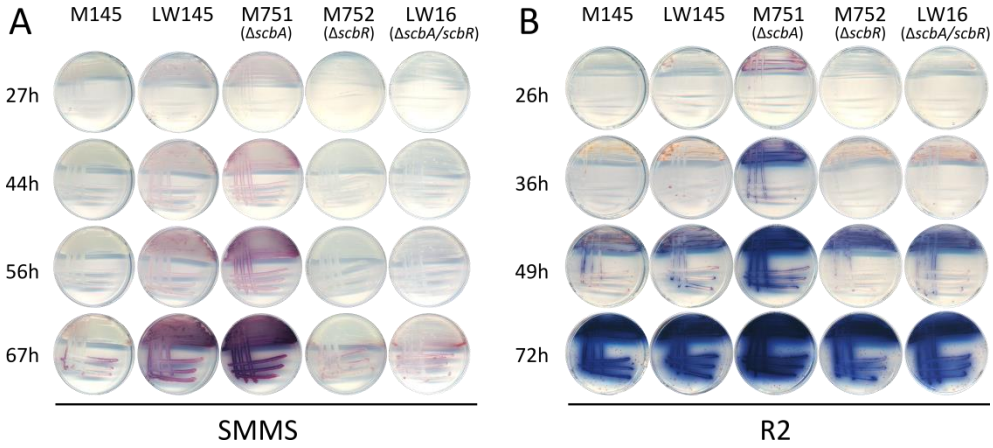


Figure 11. Antibiotic production (Act and Red) in R2 and SMMS solid media in LW145 compared to M145, M751 Δ*scbA*, M752 Δ*scbR*, and the LW16 (*scbA/scbR* double deletion mutant). Spores of the different *S. coelicolor* strains were streaked out on R2 and SMMS agar. The pink coloration corresponds to Red production and blue to the production of Act.

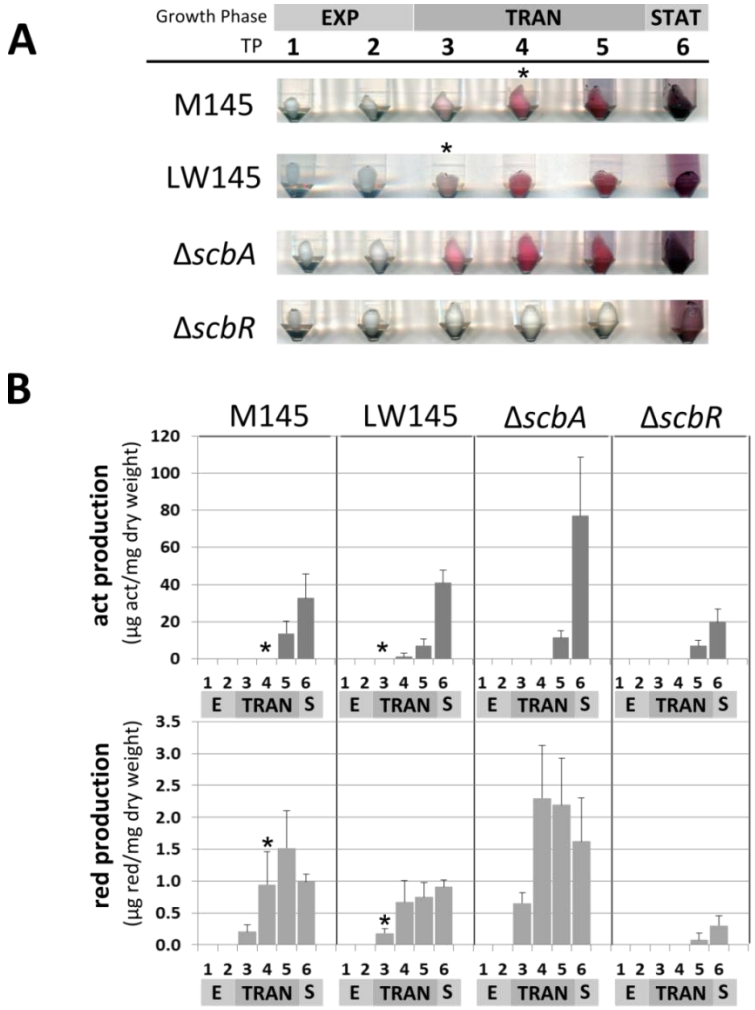


Figure 12. Comparison of Act and Red production in liquid medium between the wild type M145, the site A mutant (LW145), M751 (Δ*scbA*) and M752 (Δ*scbR*). A, The figure shows cell pellets from 1 ml of culture of each sample collected at the different time points (TP, numbers 1 to 6 in Figure 2). The production of Act and Red can be visualised by the purple and pink coloration of the pellets or the supernatant. B, Production of Act and Red was measured spectrophotometrically from the supernatants of the samples shown in A. The results show the average quantity obtained from two biological replicates. The corresponding growth phases are indicated as E, exponential phase, TRAN, transition and S, stationary phase. Asterisks indicate the onset of γ-butyrolactone production in M145 and LW145.

Discussion

The regulation of the SCB signalling system in *S. coelicolor* is complex. ScbR represses its own transcription by binding to the DNA in site R, upstream of its promoter region (see Figure 1). SCBs activate expression of *scbR* by binding to ScbR and preventing it from binding to the DNA at its binding site(s). ScbR also binds to site A, in the promoter region of *scbA*, encoding for SCB synthase. In this work, mutating site A to prevent ScbR binding resulted in early and reduced transcription of *scbA* and, in agreement with this, an early and reduced production of SCBs by LW145 compared to the wild type strain M145. Our data indicate that ScbR has a role in repressing expression of *scbA* and is involved in controlling the induction of *scbA* expression via site A in a growth phase-dependent manner.

It has been hypothesised that ScbR controls the timing of expression of *scbA*, by binding to site A and blocking its transcription (Takano *et al.*, 2001). Indeed, an increase in the expression of *scbA* in exponential phase of growth was detected in the site A mutant, where *scbR* is unable to bind to site A, as well as in a *scbR* deletion mutant. Our results thus confirm the role of ScbR in repressing *scbA* expression in exponential phase. In addition, expression of *scbA* was not induced in transition phase in the site A mutant LW145, which also indicates that ScbR has a role in controlling the growth phase–dependent induction of *scbA* transcription, through this site A. However, the exact activation mechanism of *scbA* expression is still unknown. Induction of *scbA* transcription is not solely explained by the absence of repressor protein ScbR binding to site A, and it seems more plausible that additional factors or proteins are involved. In fact, ScbA has also been reported to be essential for induction of its own gene expression (Takano *et al.*, 2001). According to this and our data, both ScbR and ScbA are essential for induction of expression of *scbA* and an interaction between these two proteins to activate *scbA* transcription is thus likely. In fact, this ScbA/ScbR interaction has been reported to be essential to maintain the bistability characteristic of the SCB system genetic switch for production of the CPK antibiotic, according to a mathematical model of the SCB system developed by Mehra *et al*, 2008. In the model this system can switch from an ON to an OFF state regarding antibiotic production depending on the concentration of SCB1, the most abundant γ-butyrolactone in *S. coelicolor*, and the concentration of free (not bound to the

γ-butyrolactone) ScbR. The behaviour of the system in the model, agrees well with our experimental data. Using gel retardation assays we failed to detect ScbA-ScbR interactions under the conditions tested (data not shown). The conditions were optimised for ScbR (according to (Takano *et al.*, 2001)) but might not be optimal for ScbA. Further experimentation will be needed to assess a possible ScbA/ScbR interaction.

Besides ScbR, several other proteins have been reported to bind to the promoter region of *scbA* and *scbR*, and could be involved in the regulation of expression of these genes, such as SlbR (Yang *et al.*, 2008, Yang *et al.*, 2012), NdgR (Yang *et al.*, 2009), SCO3201 (Xu *et al.*, 2010a), DasR (van Wezel *et al.*, 2011), and the ScbR homologous proteins CprB (Bhukya *et al.*, 2014) and ScbR2 (Wang *et al.*, 2011). ScbR2 is thought to have a role in shutting down the expression of *scbA* in stationary phase of growth, once the SCBs have carried out their signalling function in transition phase (Gottelt *et al.*, 2010, Xu *et al.*, 2010b).

It was previously reported that the *scbR* deletion mutant M752 did not produce detectable amounts of SCBs (Takano *et al.*, 2001). In this report we detected production of these signalling molecules in mutant M752 by increasing the concentration of extract tested with the kanamycin assay, as well as by LC/MS. Thus, our data demonstrate that the M752 mutant is indeed able to produce SCBs, although in considerably smaller amounts compared to the wild type. These findings indicate that ScbR is not essential for SCBs synthesis.

ScbR accumulated earlier in LW145 compared to wild type, and its levels remained high along growth in LW145 (Figure 5). This suggests that ScbR is no longer repressing its own gene expression in LW145. LC/MS analyses showed that the signaling molecules are already produced in late exponential phase of growth in the LW145 strain (Figure 7), which prevents the binding of ScbR to site R and therefore its self-repression. However, no SCBs were detected in early exponential phase, while ScbR protein was present as determined by Western blot, indicating that these signaling molecules did not yet accumulate in sufficient amounts to prevent ScbR self-repression at this growth phase. An additional factor is likely to be involved in regulating the expression of ScbR at this early stage of growth. In fact, the convergent disposition of *scbA* and *scbR* promoters suggests that transcriptional interference is taking place between

these two genes and regulating their expression. As the RNA polymerase progresses along the *scbA* gene, it might be causing occlusion to site R, or transcription factor dislodgement, making site R inaccessible for ScbR to bind while *scbA* is transcribed (Palmer *et al.*, 2011). Furthermore, a collision between the RNA polymerases transcribing from both genes is also possible (Callen *et al.*, 2004, Shearwin *et al.*, 2005), as well as the formation of an antisense RNA from the 53 bp *scbA/scbR* transcript overlap. These mechanisms have been reported to affect the bistability of the SCB system genetic switch in another mathematical model developed by Chatterjee *et al.* (2011). Their model suggests that the RNAP collision affects the bistability of the system, whereas the antisense RNA formed between *scbA/scbR* overlapping transcripts is essential to maintain it. It is possible that this *scbA/scbR* cis-antisense RNA constitutes an additional factor regulating the amount of *scbA* and *scbR* transcripts produced, that adds to the complexity of this system.

The SCBs promote the expression of *cpkO*, activator of the CPK cluster (Takano *et al.*, 2005). According to our results, the early production of SCBs observed in the LW145 mutant strain seems to lead to an early de-repression of *cpkO*, and therefore the *cpk* cluster. Unexpectedly, the production of yellow compound coelimycin P2 was delayed in the LW145 site A mutant, as was also observed for the mutant strains M752 Δ*scbR* and LW16 (Δ*scbA*/*scbR*). Since *scbR2* is also being transcribed earlier in the site A mutant, the ScbR2 transcriptional repressor, which is also a repressor of *cpkO* expression (Gottelt *et al.*, 2010, Xu *et al.*, 2010b), could be taking over the role of ScbR in blocking the expression of *cpkO* and therefore the expression of the cluster, which leads to the delay and reduction in yellow compound production observed. It is also noteworthy that CpkO and ScbR2 are not the only regulators of the CPK cluster. CpkN, a SARP transcriptional activator, whose exact role is still unknown, is situated within the CPK cluster. The complex regulation mechanism of the CPK cluster remains to be fully elucidated.

In this work, we have uncovered a growth phase–dependent role of ScbR in repressing the expression of the SCBs synthase ScbA as well as the involvement of ScbR in induction of expression of *scbA*. The mutual regulation of expression exerted by ScbR and ScbA constitutes a feedback loop to tightly regulate the coordinated expression of the *scbA* and *scbR* genes and the consequent timing of production of the signalling molecules. Our data provide more detailed

insights to better understand the complexity of the SCB system. A detailed understanding of the regulation of antibiotic production by such a signalling system will be highly beneficial in the search for new secondary metabolites that are encoded in numerous cryptic gene clusters found in streptomycetes, and may help with future attempts to engineer this system as a tool for synthetic biology (Biarnes-Carrera *et al.*, 2015).

Acknowledgements

We thank Subramaniyan Mannathan and Adriaan Minnaard from the Bio-organic Chemistry department of the University of Groningen for providing the chemically synthesised SCB1 and SCB2. Lara Martin-Sanchez was supported through a Rosalind Franklin Fellowship of the University of Groningen awarded to Eriko Takano.

Supplemental Material

Table S1. Plasmids used in this work

Name	Description	Source
pGEM-T-Easy	Cloning vector (ampicillin^R)	Promega
pCR-BluntII-TOPO	Blunt-end PCR products cloning vector (kanamycin^R)	Invitrogen
pKC1132	*E. coli/Streptomyces* shuttle vector (apramycin^R)	(Bierman *et al.*, 1992) (Kieser *et al.*, 2000)
SCAH10	Cosmid containing the *scbA/scbR* region	(Redenbach *et al.*, 1996)
pIJ6111	4.5 kb fragment from cosmid GB10 cloned in pIJ2925	(Takano *et al.*, 2001)
pIJ6114	3.1 kb *Bam*HI fragment from cosmid GB10 cloned in pIJ2925	(Takano *et al.*, 2001)
pTE74	1.9 kb DNA fragment including *scbA/scbR* region cloned in pGEM-T easy	This work
pTE75	pTE74 derivative including 4 point mutations in site A	This work
pTE76	*scbA*/*scbR* region from pTE75 subcloned in pKC1132 (containing the mutated site A and a point mutation in the *scbA* coding region)	This work
pTE76*	pTE76 containing the mutated site A and without the point mutation in the *scbA* coding region	This work
pTE31	*Bam*HI/*Xmn*I 3 kb fragment from pIJ6114 cloned in pCR-BluntII-TOPO	This work
pTE32	pTE31 with the *Bam*HI 4.5 kb DNA fragment from pIJ6111	This work
pTE33	*Hin*dIII-*Xba*I 7.5 kb DNA fragment from pTE32 cloned in pKC1132	This work
pTE34	*Pst*I-*Nru*I DNA fragment from pTE76* ligated into pTE33	This work

Table S2. List of primers used in this work

Use	Name	Sequence
Construction of site A mutant		
	Binding A	5´-CTCCTTGTTCATGTCTCCCCCGGGAAGGATA GAAAAAAAA**GGC**CTCAGTCT**C**TATCTTAACGTTC GCGCATACAGAACAGCTCGGCATCACA-3´
	BindA-fwd	5´-CTCCTTGTTCATGTCTCC-3´
	BindA-rev	5´-TGTGATGCCGAGCTGTTC-3´
	Fix-scbApm_pTE76_F	5'-GAGTCGCGAAGTCGCCGCGCATCCGCCGGTAGACTTG-3'
	Fix-scbApm_pTE76_R	5'-CAAGTCTACCGGCGGATGCGCGGCGACTTCGCGACTC-3'
	RB5F	5´-CCTCTAGATCAGCCGGAGAAC-3´
	RB5R	5´-CCGACGATATCGCCGACGTGG-3´
	RCseq25	5'-CCGTTCTCCTTGGCCTGGTTC-3'
	scbRrt1	5´-CAGGATGTGCTTCTGCAGCAG-3´
	SLIM1	5´-GCAGGCATGGCCAAGCAGGAC-3´
	SLIM2	5´-TGATTCCTGGGGGGGACCCATG-3´
	VerifA-fwd	5´-TTAAGATA**G**AGACTGAG**GCC**-3´
Gel retardation assays		
	ETS2	5'-CAAAACTACTGCTTCGGGCATG -3'
	ETS4	5'- ATCGCCCGGTCCTGCTTGGCCATG -3'
	ETS10	5'-CTTCGGTATCCAGCTGACCGGGA -3'
qRT-PCR experiments		
	AqrtF	5'- GCGCATATACAGAACAGCTC-3'
	AqrtR	5'- GATCGAGTTGGCATCGGAC-3'
	cpkOrtfw	5'-ACGATGTGGCCGGAACTC-3'
	cpkOrtrv	5'-GCCGCACCGCAGCTT-3'
	hrdBrtoutfw	5'-CATGCGCTTCGGACTCA-3'
	hrdBrtoutrv	5'-ACTCGATCTGGCGGATG-3'
	RqrtF	5'-TCATGTGATGCCGAGCTG -3'
	RqrtR	5'-CATGCCTGCCTCCTTGTT -3'
	scbR2rtfw	5'-CGGTGCTCCGGCAGATC-3'
	scbR2rtrv	5'-CCGCAGCACGACATCGT-3'

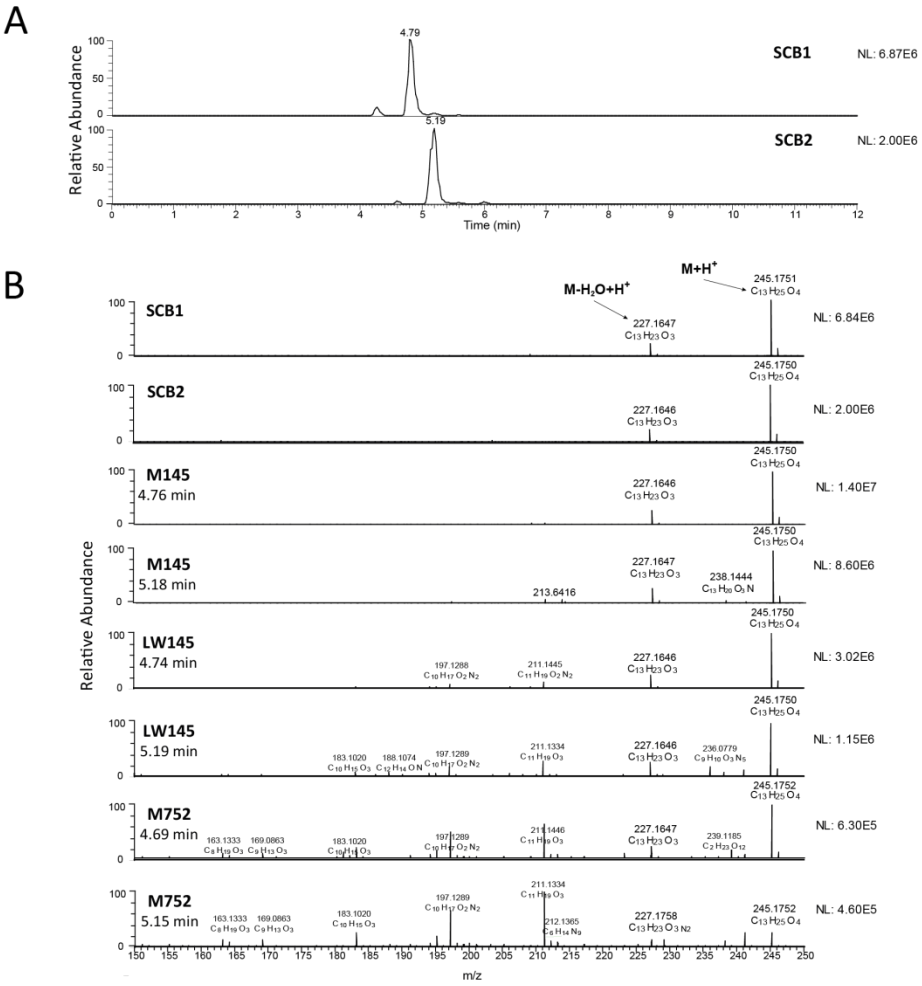


Figure S1. A, Extracted Ion Chromatogram of the chemically synthesised SCB1 and SCB2 used as standards. Pure standards of SCB1 and SCB2 are eluted at 4.79 and 5.19 min, respectively. **B, Mass spectra of SCB1 and SCB2 from the standards and biological extracts of M145, LW145 and Δ*scbR* (M752) shown in Figure 8.** SCB1 and SCB2 are detected with the correct expected masses and predicted formula: $M+H^+$:245.17 Da ($C_{13}H_{25}O_4$) and the molecular ions after loss of one or two water molecules: M-H2O+H^+: 227.17 Da with formula of $C_{13}H_{23}O_3$ and M-$2H_2O+H^+$:209.16 Da with formula of $C_{13}H_{21}O_2$. For the biological extracts, the molecular ions $M+H^+$ and the two major fragments after loss of water are visible. Predicted chemical formulas for the detected ions match with the expected formula for SCB1 and SCB2: $C_{13}H_{25}O_4$ for $M+H^+$ $C_{13}H_{23}O_3$ for M-$H_2O+H^+$and $C_{13}H_{21}O_2$ for M-$2H_2O+H^+$, respectively. This confirms the identification of these compounds in the samples.

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